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**July 8, 2004**

**Annual Report**

**"Basis of Persistent Microenvironment Perturbation in Irradiated Human Mammary Epithelial Cells"**

**Introduction**

We have shown that the progeny of irradiated human mammary epithelial cells (HMEC) show a persistent phenotypic changes resulting in disrupted morphogenesis<sup>1</sup>. In the current IDEA grant, the hypothesis is that radiation-induced disruption of morphogenesis releases constraints on genomic integrity in preneoplastic cells. Thus we predict that colonies arising from irradiated cells will show increased genomic instability due to the lack of microenvironment control rather than as a direct result of DNA damage. Aim 1 will test whether a correlation exists between the altered cytoskeleton and morphogenesis and genomic instability. In aim 2, we will determine whether irradiated cells can communicate their phenotype in a fashion similar to the 'bystander effect' that occurs when unirradiated cells respond as if they were irradiated under conditions of heterogeneous radiation exposure. In aim 3, we will begin studies to test an alternative or additional mechanism in which the phenotype is perpetuated by epigenetic modifications leading to altered gene expression. Together these studies will test whether the irradiated HMEC phenotype contributes to radiation-induced genomic instability that is observed in cells generations after radiation exposure.

**Body**

In both women and rodents, exposure to high dose ionizing radiation represents a well-established carcinogen. Epidemiologic data demonstrates that there is a significantly increased risk of breast cancer in women exposed to as little as 1 Gy as a result of atomic bomb<sup>2</sup>, therapeutic<sup>3,4</sup> or diagnostic<sup>5</sup> radiation exposures. We have previously proposed that the cell biology of irradiated tissues is indicative of a coordinated multicellular damageresponse program in which individual cell contributions are directed towards repair of the tissue<sup>6</sup>. In both murine and human models of breast cancer, our studies indicate that radiation exposure modifies the microenvironment in a manner that can promote malignant progression. Our global hypothesis is that extracellular signaling and multicellular responses following radiation contribute to its carcinogenic potential.

We have demonstrated that colonies arising from irradiated HMEC exhibit a consistent phenotype consisting of inappropriate intercellular adhesion, deranged extracellular adhesion molecules, loss of gap junction proteins, and disorganized tissue-specific organization<sup>1</sup>. This phenotype is augmented by the presence of TGF- $\beta$ , which itself is rapidly and persistently activated in irradiated tissue<sup>7</sup>. These data are remarkable in that the phenotype is inherited by the daughters of individually irradiated cells, suggesting that radiation induces a heritable derangement of pathways affecting cell adhesion, ECM interactions, epithelial polarity and cell-cell communication.

We proposed that these radiation-induced changes in cell-cell and cell-ECM interactions are consistent with malignant progression. If so, we asked whether genomic stability was similarly compromised. To do so, we began investigations to determine the status of centrosomes in this model. Centrosomes are tiny organelles that contain discrete protein aggregates that nucleate microtubule growth, organize spindle functions, and provide docking sites for protein complexes involved in cell cycle progression, checkpoint control and epithelial cell polarization (reviewed in<sup>8</sup>). Centrosomes are abnormal in both number, size and distribution in many solid tumors<sup>9</sup>. Abnormal centrosomes result in abnormal chromosome segregation and aneuploidy. Two models of centrosome involvement in cancer are currently debated. The first holds that centrosome

abnormalities, like chromosome aberrations, are the product of genomic instability; the second is that centrosome abnormalities drive genomic instability. The latter model is consistent with recent report that abnormal centrosomes precede morphological changes in transformation by HPV E7 oncoprotein<sup>10</sup> and that overexpression of pericentrin, a component of centrosomes, induces chromosome instability in prostate cancer cells<sup>9</sup>.

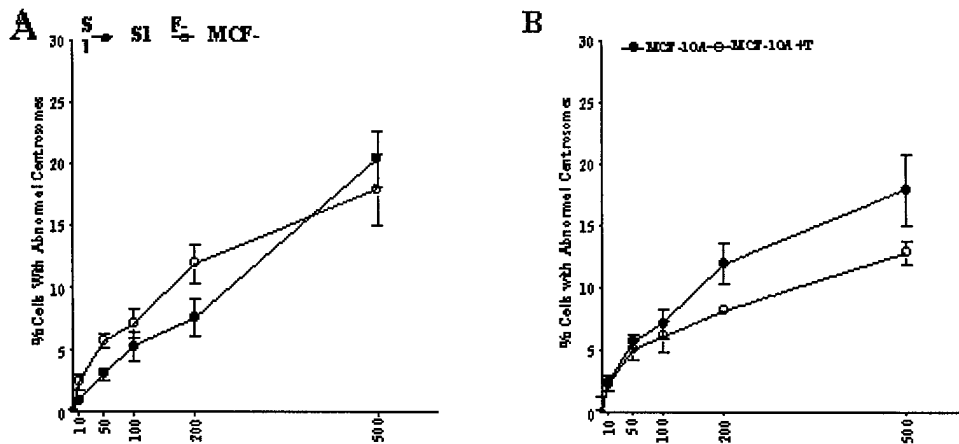
#### **Key Research Accomplishments**

- Replicated radiation dose response of induced centrosome abnormalities in HMT3522 S1 HMEC (n=3)
- Demonstration that MCF10A HMEC also show radiation dose response of centrosome abnormalities (n=3)
- Demonstrated that exogenous TGF $\beta$  suppresses abnormal centrosome induction by radiation
- Demonstrated that neutralizing antibody to TGF $\beta$  increases the frequency of centrosome abnormalities in both control and irradiated HMEC
- Demonstrated that keratinocyte cell lines from TGF $\beta$ 1 knockout mice have high levels of centrosome abnormalities compared to TGF $\beta$ 1 heterozygote cells.
- Demonstrated that abnormal centrosomes are increased following passage of irradiated cells, which indicates that this population is survival competent
- Re-evaluated double-labeling scheme for co-culture of irradiated and non-irradiated cells and developed new experimental scheme

Last year we reported that S1 cells demonstrate a dose dependent increase in the number of cells containing 3 or more centrosomes. This year our focus has been on defining the centrosome alterations and regulation by radiation-induced TGF $\beta$ , and we are currently preparing the data for publication. The work described below was carried out by Rishi Gupta, an undergraduate from the University of California at Berkeley, under the direct supervision of Dr. Anna C. Erickson, a postdoctoral fellow funded by DOD-BCRP training grant (P.I.: M.J. Bissell, LBNL) and by Dr. Rick Schwarz.

These studies were carried out on HMEC grown on tissue culture plastic rather than embedded in Matrigel since we have shown in complementary studies that the radiation-induced phenotype is evident in monolayer as well (Erickson and Barcellos-Hoff, unpublished data). HMEC were seeded onto chamber slides. On day 0, the cells were irradiated within 4 hours of plating using <sup>60</sup>Co  $\gamma$ -radiation and harvested at day 6 for staining  $\gamma$ -tubulin, a centrosome component. After immunofluorescence images were acquired using a CCD camera, the centrosome status of the cells was determined by manual counting of digital images. Cells were categorized as having abnormal centrosomes when their centrosomes were irregular in size, shape or number (>3 centrosomes per cell). This year we completed 3 replicates of the dose response in S1 HMEC and carried out similar experiments in MCF-10A HMEC. MCF-10A cells also show a dose dependent increase in abnormal centrosomes (Figure 1a). To ask whether centrosome amplification in irradiated cells is persistent MCF-10A cells treated with +/-200cGy were trypsinized and replated. Assessment of centrosome status by  $\gamma$ -tubulin immunofluorescence showed that IR cells continued to have increased frequency of abnormal centrosomes compared to control cells.

Our hypothesis that disrupted morphogenesis is accompanied by genomic instability is supported by the finding of centrosome abnormalities in the daughters of irradiated cells. We showed that exogenous TGF $\beta$  augments the irradiated phenotype in terms of morphogenesis and in

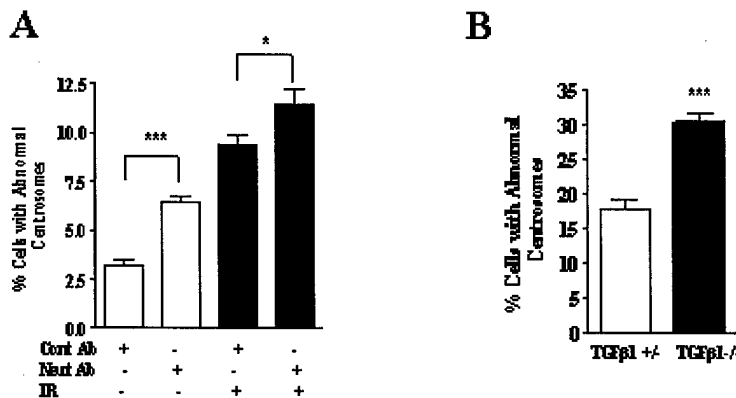


**Figure 1.** HMECs were irradiated to the indicated dose shortly after plating, grown under standard conditions and fixed at day 6. **(A)** S1 (closed circles) and MCF-10A (open circles) **(B)** MCF-10A cells mgrown in the absence (closed) or presence of 400 pg/ml TGF- $\beta$  (open circles).

he monolayer culture model. Thus, we asked whether addition of TGF $\beta$  would increase instability. Studies carried out in S1 and MCF-10A cells show that when cells are grown in the presence of TGF $\beta$  the number of cells containing abnormal centrosomes is reduced, which suggests that exogenous TGF $\beta$  actually protects S1 cells from radiation induced genomic instability (Figure 1b).

We have not found any literature that previously established a link between TGF $\beta$  and the centrosome. These data suggests that TGF $\beta$  safeguards cells from genomic instability byprotecting them from centrosome abnormalities. Further conformation of this idea was seen when we examined the centrosome status in keratinocytes isolated from mice homozygous or heterozygous for the TGF $\beta$  1 gene; TGF $\beta$   $-/-$  keratinocytes have more abnormal centrosomes than TGF $\beta$   $+/-$  keratinocytes (Figure 2b).

Our studies show that the disruption of extracellular interactions occurs in almost all colonies



**Figure 2.** **(A)** HMECs were Sham (white) or 2Gy irradiated (black) shortly after plating and grown in the presence of TGF- $\beta$  neutralizing Ab or control IgG. After 6 days centrosome status was assessed by  $\gamma$ -tubulin staining. **(B)** Centrosome status of TGF $\beta$ 1  $+/-$  (white) and TGF $\beta$ 1  $-/-$  (black) keratinocytes was evaluated by  $\gamma$ -tubulin staining in confluent monolayers.

formed by cells that survive irradiation. If the mechanisms by which the irradiated phenotype is perpetuated involve extracellular signaling via soluble molecules or cell contact, irradiated cells will be able to influence unirradiated cells via a "bystander" mechanism. We have now shown that TGF $\beta$  is clearly implicated. We first showed that TGF $\beta$  is activated *in vivo* after exposure to ionizing radiation<sup>11</sup>. We have now found that irradiated S1 cells also increase TGF $\beta$  signaling. We hypothesized that this radiation-induced TGF $\beta$  activity is acting in a bystander capacity to protect irradiated cells from genomic instability based on the data from irradiated HMEC treated with exogenous TGF $\beta$ . Pan-specific TGF $\beta$  neutralizing antibodies were used to test whether the endogenous TGF $\beta$  affects the number of cells containing abnormal centrosomes. S1 cells were treated with +/- 200 cGy and grown in the presence of TGF $\beta$  blocking antibodies or IgG controls. Interestingly, blocking TGF $\beta$  signaling affected unirradiated and irradiated cells equally, i.e. both treatment groups displayed an increase in abnormal centrosomes (Figure 2A). This data allowed us to draw two conclusions, firstly, radiation-induced TGF $\beta$  signaling does protect irradiated cells from genomic instability and secondly, endogenous TGF $\beta$  plays a role in controlling/regulating centrosome status in S1 cells.

To test this, we are developing a protocol to differentially label HMEC with 'CellTracker' dyes (Molecular Probes, Eugene OR) which passively diffuse into live cells. Once inside, the non-fluorescent compounds are cleaved by intracellular enzymes to yield highly fluorescent dyes. After labeling, the external dyes are removed by changing the medium. Of the two types of CellTracker dyes, a thiol or amine reactive, only the thiol group was retained in this cell type. In addition, loading the mammary epithelial cell with dye was not completely non toxic. In cells already damaged by ionizing radiation, loading cells reduced viability. Therefore, in the experiments described below, only normal cells were labeled with thiol reactive CellTracker dyes.

The original experimental approach was to start by subculturing the cells. While the cells were in suspension, they were divided into two groups. The normal group was stained for 30 minutes with a CellTracker dye; the IR group was irradiated with 2 Gy of x-rays. The cells were then plated onto multi-chamber slides B one chamber normals only, a second chamber IR only, and two chambers with varying amount of both types. We then analyzed changes in E-cadherin levels between cells and changes in centrosome number. This experimental design turns out to have several disadvantages. One, by labeling cells with dye at the time of seeding, the cells go through many rounds of cell division causing the dye to be diluted fairly rapidly. This limits the duration of the experiment to only 3 or 4 days. Two, by mixing normal and irradiated cells together at time zero, one is observing both the recovery from being subcultured, the transition to forming colonies, and Abystander@ interactions. Three, in mixed population experiments where the two groups are dispersed amongst each other, it is more difficult to resolve who is influencing whom. These problems can be remedied by change in experimental protocol.

In our new design, normal cells will be seeded at a low density and allowed to grow into small to medium colonies. The cells can then be labeled with CellTracker dye and the medium changed to clear the excess dye. Then irradiated cells will be plated at a moderate density in the same well and they will surround the normal colony. After, 4 days we will analyze changes in the normal colonies as to e-cadherin levels and centrosome number. The controls will be to add unlabeled normal cells to the dye labeled normal colonies. This design should help retain the CellTracker dye since the normal labeled cells will proliferate less. In addition, the normal cells will be in colonies so perturbations due to recovery from subculturing should be minimal. The analysis will be easier since we can focus on changes within a colony of only normal cells.

To be more physiological, we wanted to observe "bystander" effects on 3-dimensional colonies.

Barcellos-Hoff, M.H.

In the original design, it was proposed to aggregate normal and irradiated S1 cells by mixing them together and then seeding them in a reconstituted basement membrane, Matrigel. S1 cells, however, are remarkably unsticky after being trypsinized and fail to form mixed cell aggregates even when spun and resuspended at high concentration. To circumvent this problem, we found that cells will grow together in 2-dimensional cultures if plated close to each other. Then one can drip matrigel on top of the cells, causing them to form mixed 3-dimensional structures. This method should work with minor modifications to convert our new protocol to a 3-dimensional assay system. In this case, non-irradiated cells would only be grown to very small colonies (<10 cells) before the irradiated cells were added, then a Matrigel drip would be added upon feeding to observe the ability of these cells to form an organized 3-dimensional colony incorporating both normal and irradiated cells.

As reported last year, studies regarding methylation as a mechanism driving the phenotype of irradiated cells have been conducted. Studies to investigate acetylation of histones will begin shortly.



### **Reportable Outcomes**

#### *Presentations by PI*

"Non-Mutagenic Actions of Radiation in Mouse and Human Mammary Carcinogenesis", Keystone Symposia on Mouse Models of Cancer, February, 21, 2004.

"TGF $\beta$ 's Role in Mammary Gland Development and Carcinogenesis", 24th Congress of the International Association for Breast Cancer Research, Sacramento, CA, November 4, 2003.

"TGF $\beta$  Regulation of Cell Fate Decisions Following DNA Damage", Ludwig Institute for Cancer Research Uppsala, Sweden, September 26, 2003.

"Extracellular Signaling in Radiation Responses", International Congress of Radiation Research, Brisbane, Australia, August 19, 2003.

"Of Mice and Women: Modeling Radiation Carcinogenesis", Life Sciences Division, Oak Ridge National Laboratory, August 4, 2003.

"Mechanisms and Consequences of TGF $\beta$  $\beta$ 1 Activation in Mammary Gland Development and Carcinogenesis", University of Puerto Rico Cancer Center and Departments of Biochemistry and Physiology, June 9, 2003.

#### *Poster and Meeting Participation*

Erickson, A.C., Gupta, R., Parvin, B. and Barcellos-Hoff, M.H. TGF $\beta$ 1 Protects Human Mammary Epithelial Cells from Radiation-Induced Centrosome Amplification. DOE Low Dose Radiation Workshop, October 27-29, 2003.

Gupta, R., Erickson, A.C., Parvin, B. and Barcellos-Hoff, M.H. TGF $\beta$ 1 Protects Human Mammary Epithelial Cells from Radiation-Induced Centrosome Amplification. American Society for Cell Biology, San Francisco, December 9-12, 2003.

### **Conclusions**

Our current studies regarding radiation-induced phenotype and genomic instability support the widely acknowledged dual role of TGF $\beta$  in cancer<sup>12</sup>. In mouse models overexpression of active TGF $\beta$  suppresses tumorigenesis<sup>13</sup>, but converts to a tumor promoting role in late carcinogenesis in skin, mammary gland and colon<sup>14-16</sup>. In response to radiation TGF $\beta$  likewise appears to protect against genomic instability generated by centrosome amplification, while promoting phenotypic neoplastic progression. Our future studies will seek to define the molecular mechanism by which radiation-induces centrosome amplification and that by which TGF $\beta$  regulates it.

### **References**

1. Park CC, Henshall-Powell R, Erickson AC, Talhouk R, Parvin B, Bissell MJ, Barcellos-Hoff MH: Ionizing Radiation Induces Heritable Disruption of Epithelial Cell-Microenvironment Interactions. *Proc Natl Acad Sci* 2003, 100:10728-10733
2. Tokunaga M, Land CE, Tokuoka S: Follow-up studies of breast cancer incidence among atomic bomb survivors. *J Radiat Res (Tokyo)* 1991, 32 Suppl:201-211.
3. Mattsson A, Ruden B-I, Wilking N, Rutqvist LE: Radiation-induced breast cancer: Long-term follow-up of radiation therapy for benign breast disease. *J Natl Cancer Inst* 1993, 85:1679-1685
4. Mauch P: Second malignancies after curative radiation therapy for good prognosis cancers. *Int J Radiation Oncology Biol Phys* 1995, 33:959-960
5. Davis FG, Boice JD, Hrubec Z, Monson RR: Cancer mortality in a radiation-exposed cohort

- of massachusetts tuberculosis patients. *Can Res* 1989, 49:6130-6136
6. Barcellos-Hoff MH: How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues. *Radiation Res* 1998, 150:S109-S120
  7. Barcellos-Hoff MH, Derynck R, Tsang ML-S, Weatherbee JA: Transforming growth factor- $\beta$  activation in irradiated murine mammary gland. *J Clin Invest* 1994, 93:892-899
  8. Salisbury JL: The contribution of epigenetic changes to abnormal centrosomes and genomic instability in breast cancer. *J Mammary Gland Biol Neopl* 2001, In press
  9. Pihan GA, Purohit A, Wallace J, Malhotra R, Liotta LA, Doxsey SJ: Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res* 2001, 61:2212-2219
  10. Duensing S, Duensing A, Crum CP, Munger K: Human papillomavirus type 16 E7 oncoprotein-induced abnormal centrosome synthesis is an early event in the evolving malignant phenotype. *Cancer Res* 2001, 61:2356-2360
  11. Barcellos-Hoff MH: Radiation-induced transforming growth factor  $\beta$  and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res* 1993, 53:3880-3886
  12. Roberts AB, Wakefield LM: The two faces of transforming growth factor {beta} in carcinogenesis. *PNAS* 2003, 100:8621-8623
  13. Pierce DF, Gorska AE, Chytil A, Meise KS, Page DL, Coffey Jr. RJ, Moses HL: Mammary tumor suppression by transforming growth factor  $\beta$ 1 transgene expression. *Proc Natl Acad Sci USA* 1995, 92:4254-4258
  14. Cui W, Fowles DJ, Bryson S, Duffie E, Ireland H, Balmain A, Akhurst RJ: TGF $\beta$ 1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* 1996, 86:531-542
  15. Oft M, Heider K-H, Beug H: TGF $\beta$  signaling is necessary for carcinoma cell invasiveness and metastasis. *Current Biology* 1998, 8:1243-1252
  16. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E: TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996, 10:2462-2477